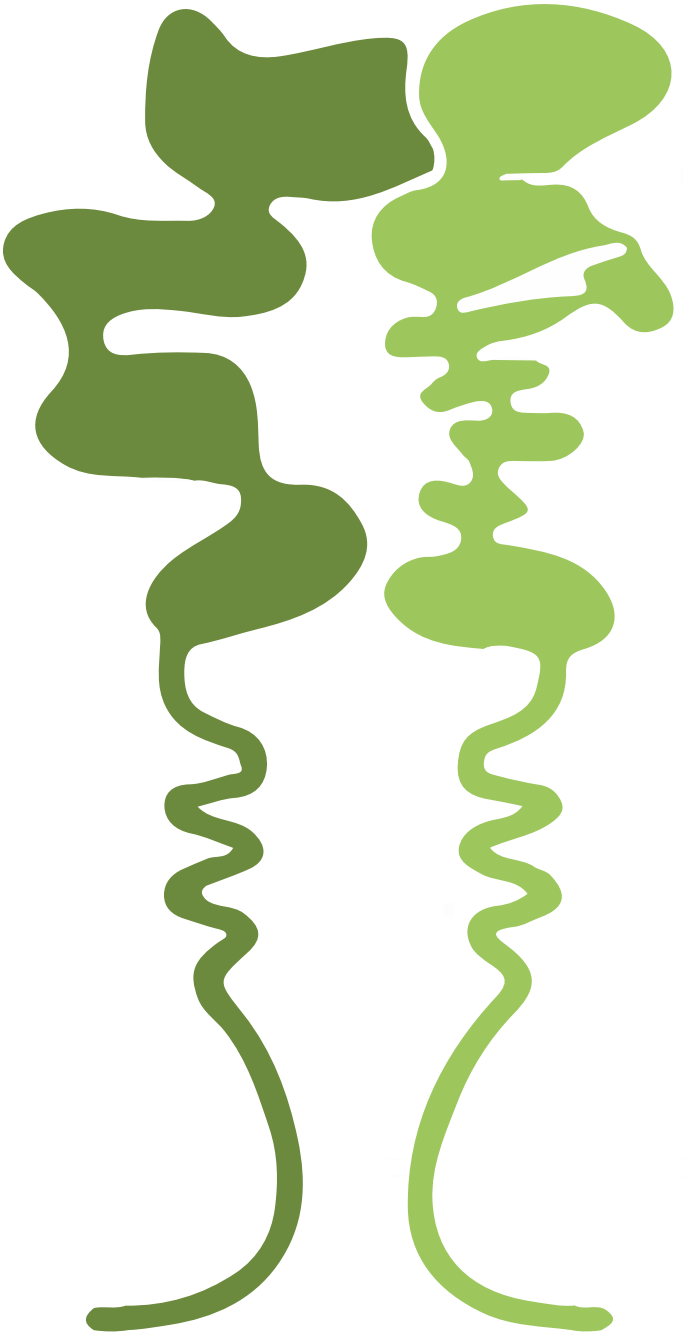
The diagnostic value of plasma thrombopoietin levels and platelet autoantibodies

Porcelijn, L.



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CHAPTER 5

Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art

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Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art.

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Summary

Immune Thrombocytopenia (ITP) is diagnosed by exclusion of other causes for thrombocytopenia. To prevent misdiagnosis, reliable detection of platelet autoantibodies would support the clinical diagnosis. We optimized our diagnostic algorithm for suspected ITP using the direct monoclonal antibody immobilization of platelet antigens (MAIPA) assay. In this test, the presence of platelet autoantibodies on the glycoproteins (GP) IIb/IIIa, Ib/IX and V bound on the patient platelets, is evaluated. The direct MAIPA showed to be a valuable technique for the detection of platelet autoantibodies and can possibly become a guide for optimizing therapy towards a more personalized treatment of ITP.

Platelet autoantibodies are regarded to be the major underlying cause of immune thrombocytopenia (ITP), although a role for cytotoxic T cells is also described (Cines et al, 2014). Screening for platelet autoantibodies however, is not part of the recommended diagnostic and therapeutic work up (Neunert et al, 2011). The latter is due to so far low sensitivity (60-70%) and specificity (≤ 60%) of the different types of platelet autoantibody tests (Hagenström et al, 1983; Helmerhorst et al, 1983) and ITP is therefore diagnosed by exclusion of other causes for thrombocytopenia (Neunert et al, 2011). To prevent misdiagnosis, reliable detection of platelet autoantibodies however, would be of great value for the clinical diagnosis. In this respect, we re-evaluated our diagnostic algorithm for suspected ITP using the direct monoclonal antibody immobilization of platelet antigens assay (MAIPA).

Platelet autoantibodies in ITP are predominantly directed against the platelet glycoproteins (GP) IIb/IIIa (CD41/61; fibrinogen receptor), GPIb/IX (CD42c/CD42a) or GPV (CD42d) (Joutsi & Kekomäki, 1997; McMillan, 2003). The presence of platelet antibodies directed against any of these targets can be investigated by ELISA-based GP specific assays, such as MAIPA and Luminex beads assays (Kiefel et al, 1987; Porcelijn et al, 2014). While the indirect MAIPA and commercially

available GP specific assays are known for their high sensitivity and specificity for identification of human platelet antigen (HPA)-specific allo-antibodies (Porcelijn et al, 2008), platelet autoantibodies in ITP serum or plasma are less easy detected (McMillan, 2003). Also the direct MAIPA, developed to directly detect platelet-bound antibodies, showed in previous studies a sensitivity for autoantibodies ranging from only 29 to 54% (Joutsi & Kekomäki, 1997; McMillan, 2003).
A more accurate detection of platelet autoantibodies in this respect would be of great value and for this reason, we validated detection of platelet autoantibodies by the direct MAIPA in known ITP and non-ITP mediated thrombocytopenic patients as well as non-thrombocytopenic controls. Subsequently, we tested the direct MAIPA for its discriminatory power between ITP and non-ITP patients in consecutively diagnostic samples sent to our laboratory.

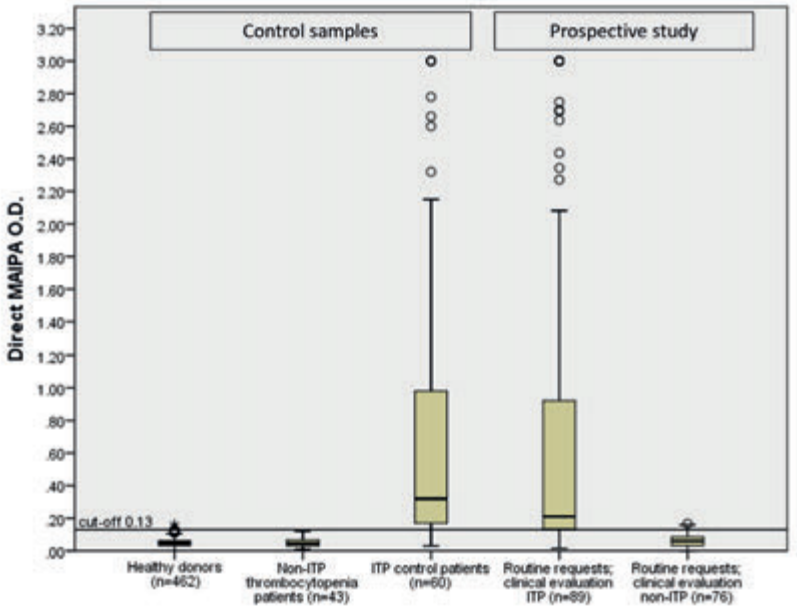
Healthy control and patient platelets, platelet eluates and sera were tested, within 24 hours after sampling, with the direct and indirect platelet immunofluorescence test (PIFT) (as described by von dem Borne et al, 1978). for the presence of platelet-associated and free circulating autoantibodies of the immunoglubolin (Ig)G- and IgM-class, and with the direct MAIPA (as described by Kiefel et al, 1987), for the presence of the IgG-class platelet-associated autoantibodies.
Statistical analyses were performed using SPSS 21 for Windows statistical package (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered significant.
Healthy control (n=462) platelets, tested with the direct MAIPA, produced a range of normally distributed very low extinctions (Fig 1, between E=0.048 and E=0.052 (range 0.01 – 0.16 ± 0.023 – 0.026) for all five autoantibody targets GPIIb/IIIa (CD41/CD61), GPIb/IX (CD42c/CD42a), GPV (CD42d), GPIa/IIa (CD49b/CD29) and GPIV (CD36)). With the calculated cut-off value E = 0.13 (mean + 3 x standard deviation (SD)) only one of the healthy controls showed a positive (O.D. 0.137) direct MAIPA result for only CD41/61 (GPIIb/IIIa) (specificity of 99.8%: Fig 1, Table I).

Six of the 462 healthy controls showed positive results in the direct PIFT by the presence of antibodies of the IgM-class.
None of the non-immune thrombocytopenic patients (n=43, Supplemental Table Ia) showed a positive direct MAIPA result, whereas 16 of these 43 (37%) non-immune thrombocytopenia samples were positive, both with direct PIFT and eluate PIFT (Table I).

Known ITP patients (n=60) were diagnosed - in accordance with the recommendations of the American Society of Hematology (Neunert et al, 2011) - by means of the medical history, physical examination, complete blood count, the peripheral blood smear, platelet counts between 10 and 50 x 109/L, normal or slightly increased plasma thrombopoietin levels (as described by Folman et

al, 1997) and did not receive treatment for at least 3 months. The direct MAIPA produced positive reactions for 51/60 (85%) of these known ITP patients (Fig 1, Table I). The PIFT (direct + eluate) produced positive reactions in only 39 (65%) of these samples. Most autoantibodies were directed against GPIIb/IIIa, GPIb/IX and/or GPV. None of the samples had autoantibodies exclusively directed against GPIa/IIa and/or GPIV detected (Table SII).

For 178/204 (86%) thrombocytopenic patients suspected for ITP, the MAIPA was performed without knowledge of the clinical diagnosis. For 26 of these patients, all with a platelet count < 10 x 109/L, an insufficient number of platelets could be isolated. Clinical data was obtained for 165 of the remaining 178 tested patients (Figure S1). ITP was excluded based on the clinical data in 76 of these 165 patients (46%) with a mean platelet count of 104 x 109/L (range 9-386, SD 71) and a mean plasma Tpo level of 106 AU/ml (range 5-956, SD 166; Figure S2). In 25 of these patients, the platelet counts never fell below 100 x 109/L (Table SIB). Seventy-four of these 76 patients (97%) had a negative direct MAIPA result (Figure 1). Of the two patients with a positive direct MAIPA result, one



Direct MAIPA results for ITP patients and controls.
Direct MAIPA O.D. above 0.13 is considered positive. Control samples: historically well characterized ITP patients. Prospective study: requests for serological ITP diagnostics, after final clinical evaluation classified as ITP or non-ITP.

suffering from autoimmune haemolytic anaemia; showed GPV (CD42d)-bound platelet autoantibodies (O.D. 0.199), PIFT negative. The other patient, diagnosed with EDTA-dependent pseudothrombocytopenia (platelet counts in EDTA- and citrate-anticoagulated blood respectively, 60 and 109 x 109/L) showed GPV- and GPIb/IX-bound platelet autoantibodies (O.D. 0.176 and 0.186); the direct PIFT was weak positive for IgM only; the eluate PIFT was negative.

Table I. Test results for: healthy donors (n=462), ITP patients (n=60), non-ITP patients (n=43) and prospective requests for ITP diagnostics (n=165).

	Direct MAIPA positive Direct PIFT* positive n(%)	Direct MAIPA positive Direct PIFT* negative n (%)	Direct MAIPA negative Direct PIFT* positive n(%)	Direct MAIPA negative Direct PIFT* negative n(%)
Controls (n=545)				
Healthy donors (n=462)	0	1 (0.2%)	6† (1.3%)	455 (98.5%)
Non ITP (n=43)	0	0	16 (37%)	25 (58%)
ITP (n=60)	39 (65%)	12 (20%)	4 (7%)	5 (8%)
Routine requests (n=165)				
ITP (n=89)	60 (67%)	9 (10%)	2 (2%)	18 (20%)
Non-ITP (n=76)#	2 (3%)	1 (1%)	26 (34%)	47 62%)
Total group (n=268)				
ITP (n=149)	99 (66%)	21 (14%)	6 (4%)	23 (15%)
Non-ITP (n=119)#	2 (2%)	1 (1%)	44 (37%)	72 (61%)

ITP, immune thrombocytopenia; MAIPA, monoclonal antibody immobilization of platelet antigens assay; PIFT, platelet immunofluorescence test.
**PIFT= direct PIFT + eluate PIFT, †all six positive results were due to antibodies of the IgM class, # By clinical data analysis, ITP could be excluded for 76 of the 165 patients, initially suspected for ITP.*

In 89 of the 165 tested samples, the diagnosis of ITP was clinically made, the mean platelet count in this group was 45 x 109/l (range 8-171, SD 34.7). A mean plasma Tpo level of 38 AU/ml (range 4-381 AU/ml, SD 62 AU/ml; Figure S2)) was found. The direct MAIPA was positive for 69/89 (78%) of these patients (Figure 1, Table I).

Platelet associated antibodies of the IgG (and/or IgM) class were detected in the direct PIFT in 62/89 (70%) suspected ITP patients; for two of these, no antibodies were detected by the direct MAIPA.

Overall, the direct MAIPA correlated with the clinical diagnosis of ITP with a sensitivity of 81% (95% CI, 73-87%), and a specificity of 98% (95% CI, 94-100%). A positive predictive value of 98% (95% CI, 94-100%) for clinical ITP and a negative predictive value of 80% (95% CI, 72-86%) were obtained.

The direct MAIPA has two limitations. First, from approximately 16% of the routine ITP diagnostics referred samples insufficient patient platelets can be isolated to perform a direct MAIPA. Second, no autoantibodies are detected in approximately 20% patients suspected for ITP. Intriguingly, this lack of antibodies might still be considered as an immune dependent thrombocytopenia i.e. caused by T-cell autoimmunity. Additional research in these clinically typical ITP patients without detectable antibodies should reveal the nature of such thrombocytopenias.

Notwithstanding the limitations, advantages of the direct MAIPA assay are many-fold. Next to its value for enabling a much more reliable ITP diagnosis, the presence and further characterization of the glycoprotein specificity of platelet autoantibodies in the direct MAIPA assay may be correlated with the severity of bleeding symptoms and additionally lead to a more personalized ITP therapy. For instance, autoantibodies blocking the fibrinogen binding-site of GPIIb/IIIa were found associated with more severe bleeding in ITP (De Cuyper et al, 2013). Furthermore, platelet autoantibodies binding to platelet GPIb/IX, have been shown to induce desialylation of GPIb/IX and as such more prevalent destruction of the platelets by the Ashwell-Morell receptor of hepatocytes (Li et al, 2015). If so, such findings would make intravenous Ig treatment and splenectomy less likely effective. Third the inhibitory effect of platelet autoantibodies on compensatory thrombocytopoiesis might also depend on the glycoprotein specificity of the antibodies and less response of these patients to Tpo analogues (Iraqi et al, 2015). Finally, in our assays, the observed changes in antibody presence might steer the continuation, tapering or stopping of treatment. Our recently described association of antibody presence and lowering thereof after rituximab nicely underlines the value of our assays (Porcelijn et al, 2017).

We conclude that the direct MAIPA not only enables a more reliable diagnosis of ITP but may also help in the choice and continuation of therapy i.e. by monitoring the immune activity in ITP during long term TPO analogues. This in the end will be indispensable for more personalized treatment algorithms for ITP.

Authorship

L.P. conceptualized and designed the study, conducted the data analysis and statistical analysis, drafted the initial manuscript, and approved the final manuscript as submitted.

E.H. and G.O. coordinated and supervised data collection, critically reviewed the manuscript and approved the final manuscript as submitted.
M.S. reviewed and revised the manuscript and approved the final manuscript as submitted.

J.J.Z. and M.de H. supervised the study, conceptualized and co-drafted the initial manuscript, and approved the final manuscript as submitted.
None of the authors has a financial conflict of interest.

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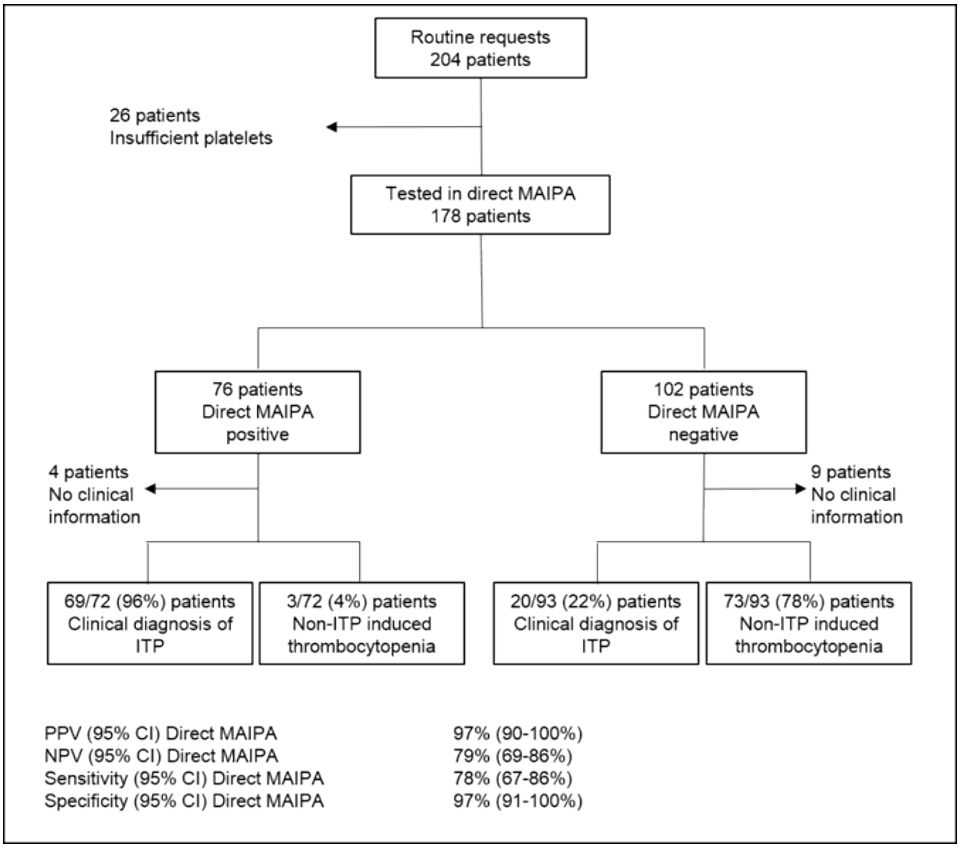
Supplemental data:
Detection of platelet autoantibodies revisited to identify immune thrombocytopenia.



Supplemental Figure 1: *correlation direct MAIPA results and clinical diagnosis*
PPV positive predictive value, NPV negative predictive value, CI confidence interval

Supplemental Table Ia. non-ITP thrombocytopenic patients (n=43)

non-ITP thrombocytopenia patients	n=43	Platelet counts x 10^9/L
hematological malignancies	n=17	ranging from 10-131
gestational thrombocytopenia	n=7	100, 107, 111, 119, 121, 124 and 130
viral infections	n=6	66, 83, 110, 120, 134 and 147
drug-induced thrombocytopenia	n=4	54, 77, 85 and 100
aplastic anemia	n=3	9, 87 and 100
hepato-splenomegalic pooling	n=3	50, 60 and 123
pseudothrombocytopenia	n=2	in EDTA and citrate; 50, 70 and both > 150, respectively
microangiopathy	n=1	20 x 10E9/L

Supplemental Table Ib. Routine ITP serology request patients, ITP excluded* (n=76)

Total	*n=76*	Mean platelet count 94 x 10^9/L, range 9-386, STD 50
hematological malignancies	n=24	ranging from 9-124
gestational thrombocytopenia	n=13	ranging from 69-134
drug-induced thrombocytopenia	n=9	ranging from 9-147
erroneous requests by administrative errors	n=9	all > 150
pseudothrombocytopenia	n=5	in EDTA and citrate; ranging from 33-102 and 109-231, respectively
aplastic anemia	n=5	64, 72, 88, 106 and 113
hepato-splenomegaly	n=4	45, 63, 71 and 73
bacterial sepsis	n=3	12, 24 and 31
anti-phospholipid syndrome	n=1	36
thrombotic thrombocytopenic purpura	n=1	99
congenital thrombocytopenia	n=1	12
post cardiac infarct	n=1	133

** By clinical and laboratory data analysis, ITP could be excluded for 76 of the 165 patients, initially suspected for ITP.*

Supplemental Table IIa: Pattern of reactivity of platelet autoantibodies in ITP samples, as determined with the direct MAIPA.

	GPIIb/IIIa+ GPIb/IX+ GPV	GPIIb/IIIa+ GPIb/IX	GPIIb/IIIa+ GPV	GPIb/IX+ GPV	GPIIb/IIIa	GPIb/IX	GPV	negative	total
Number of samples reactive with	18 (30%)	10 (16.7%)	6 (10%)	4 (6.7%)	6 (10%)	4 (6.7%)	3 (5%)	9 (15%)	60 (100%)

Supplemental Table IIb: Platelet autoantibody reactivity in ITP samples as tested for five platelet glycoproteins with the direct MAIPA.

	GPIIb/IIIa	GPIb/IX	GPV	GPIa/IIa	GPIV
Number of samples reactive with	4 0 / 6 0 (66.7%)	3 6 / 6 0 (60%)	3 1 / 6 0 (51.7%)	13/32 (40.6%)	7/26 (26.9%)

Supplemental Figure 2: *Platelet counts versus plasma Tpo levels for routine request patients*